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Viral load and not Lactoferrin concentrations in breast milk correlates with the transmission of Human Cytomegalovirus (HCMV) to preterm neonates.

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ABSTRACT

In vitro, lactoferrin (LF) strongly inhibits Human Cytomegalovirus (HCMV), which led us to hypothesise that *in vivo* HCMV might also be inhibited in secretions with high LF concentrations. In breast milk, high viral loads observed as high viral DNA titers, tended to coincide with higher LF levels. However, the LF levels did not correlate to virus transmission to preterm infants. Viral load in the transmitting group was highest, as compared to the non-transmitting group. We conclude that viral load in breast milk is an important factor for transmission of the virus.

INTRODUCTION

Breast-feeding is a strong risk factor for the postnatal transmission of Human Cytomegalovirus (HCMV) [21,2]. The rate of transmission by consuming HCMV-infected breast milk ranges from 58 to 76% [3,24].

Although HCMV infected cells have been isolated from breast milk [1,5], and cell free virus has been detected in whey of HCMV infected mothers [1,5], the mechanism of virus transmission through breast milk has not been elucidated yet. In contrast, HCMV is seldomly detected in colostrum [16]. Breast milk has a protective effect against microbial infections and one of the protective components is lactoferrin (LF).

Lactoferrin, an 80 kD ironbinding glycoprotein, is present in the secondary vesicles of neutrophilic granulocytes [12]. Lactoferrin is also present in mucosal secretions [11,13] where it is produced by epithelial cells e.g. by the mammary glands during lactation [11,13]. At the mucosa, LF exerts its antibacterial and fungicidal effect [10,11,13]. *In vitro*, LF exerts antiviral activities against a plethora of viruses, including hanta, HIV and HCMV [6,15,18,22].

Lactoferrin concentrations are highest in colostrum and tend to decrease significantly within the first weeks of lactation [7,14]. We hypothesised that LF, among other defence proteins, would help to prevent the transmission HCMV to the newborn. In particular for preterm new-borns this non-specific immunological defence could be important.

We set out to determine the LF concentrations in breast milk longitudinally, to assess the relation between transmission of HCMV and LF levels *in vivo*. The relation between LF concentrations and the total amount of HCMV DNA in breast milk was studied in the same samples.

MATERIALS & METHODS

Study group.

Breastmilk specimens were obtained from 23 breast-feeding mothers of preterm infants at the University Hospital of Tübingen. These mothers were enrolled prospectively between July 1995 and June 1998 in a clinical study of postnatal mother-to-preterm infant transmission of HCMV by breast milk [4]. HCMV screening of seronegative and seropositive mother-infant pairs was performed by serology, virus culture and PCR. Congenital and perinatal HCMV transmission were excluded. All mothers were informed of the aim of the study, which was approved by the ethical committee of the University of Tübingen. All mothers were without clinical symptoms of HCMV infection and were classified into 4 groups.

Seronegative controls (group 1, n = 4) i.e. without transmission, DNA- and virolactia. Groups 2 (n = 4), 3 (n =8) and 4 (n = 7) all comprised seropositive mothers with DNA-lactia. Transmission only occurred in group 4 of which the mothers, like in group 3, had virolactia. Group 2 mothers had no virolactia.

Milk whey preparation.

Native expressed breast milk was sampled longitudinally. Cell free milk whey was prepared as described previously [5] and stored aliquotted at -20°C.

DNA extraction and qualitative nPCR from milk whey.

The extraction of DNA and detection of HCMV DNA by nPCR in milk whey was performed as previously described [5]. This approach allowed detection of 200 Genome Equivalents (GE) x ml^{-1} from milk whey.

Determination of viral load by quantitative nested PCR (qnPCR).

Extracted DNA from breast milk samples were added to PCR reaction mixtures containing 50 copies (high standard) or 10 copies (low standard) of a cloned CMV standard [9,17]. Target sequences were amplified with the external CMV-specific primers E1 and E2 [17]. Five microliters each of the external reaction

was reamplified in a second round of PCR with the internal CMV-specific primers TGGE1B and TGGE2E. Standard and wild type CMV PCR amplimers were quantitated by hybridisation analysis as described [17]. For CMV DNA copies ≥20 in 2.5 µl the data from the high-standard reaction were used, and for CMV copies <20, data from the low-standard reaction were used. Results were expressed as the number of CMV wild-type genome equivalents (GE) per ml of milk whey. Exact quantification was possible between 400 to 200,000 GE/ml.

Detection of virolactia and transmission.

HCMV was cultured from milk whey by using HFF (Human Foreskin Fibroblasts) in the tube cell culture system. Virus transmission to the preterm infant was documented by positive viruria or DNA-uria not earlier than three weeks after delivery. Viruria was detected by virus culture, DNA-uria by nPCR as outlined above.

Quantification of LF levels in breast milk.

Lactoferrin concentrations in breastmilk were determined as described [23], with minor modifications. In brief, polyclonal antiserum against human LF (Jackson, USA) was coated in 96-wells plates (Hycult, The Netherlands). Serial dilutions of breast milk were added to the wells. Human LF (Sigma, USA) was used in the calibration curve. Bound antibody was detected with horse-radish-peroxidase labelled antibodies (Jackson). Colour was developed with TMB (Tri-Methyl Benzidine, Sigma) and the optical density (OD) at 450 nm was measured. These optical densities were converted to LF concentrations using a four parameter curve-fitting algorithm.

Statistical analyses.

The courses of LF concentrations in breast milk were calculated using smoothing spline fits. Unpaired t-tests were performed to investigate differences in HCMV DNA levels between the transmitting and non-transmitting groups.

RESULTS & DISCUSSION

In milk whey of breastfeeding mothers of preterm infants, LF concentrations were maximal in colostrum, up to 7 mg/ml, and decreased approximately sevenfold in two weeks time (Fig. 1). This is consistent with reported values found during term delivery [7,14]. Although, for 4 mothers an increase in LF levels was observed.

A significant difference in initial breast milk LF concentrations, or decline in LF concentrations during the course of lactation between the 4 different groups was not observed. The individual LF levels of the mothers in the transmission group tended to be more variable when compared to the other groups (Fig. 1). According to the spline fits however, these variations did not reach statistical significance. A significant correlation between lactoferrin concentrations and the amount HCMV DNA in breast milk was not observed (data not shown).

Viral loads in whey of HCMV transmitting mothers (group 4) were significantly higher ($p = 0.024$) as compared to non-transmitting mothers (groups 2 and 3, Fig. 2). Transmission of HCMV to new-borns was observed only above a viral load of approx. 7×10^3 GE/ml. Thus. Above this threshold not even high LF levels appear to protect from transmission. Indeed, we showed that the transmission of cell-bound virus *in vitro* could only be inhibited for 50% [8].

The reason for the more variable breast milk LF concentrations in the transmitter group could be reflected by different degrees of local inflammation in the breast [13]. It is conceivable that, when large amounts of virus are present in breast milk, there also is viral replication in the breast, leading to a local inflammation reaction. As a result of this inflammation, the viral load in the transmission group (group 4) could have increased above a threshold level, which would lead to transmission and primary infection of the newborn. Although *in vitro* and *in vivo* data show that HCMV can replicate in several cell types [19,20], the exact replication site in the mammary gland is not known.

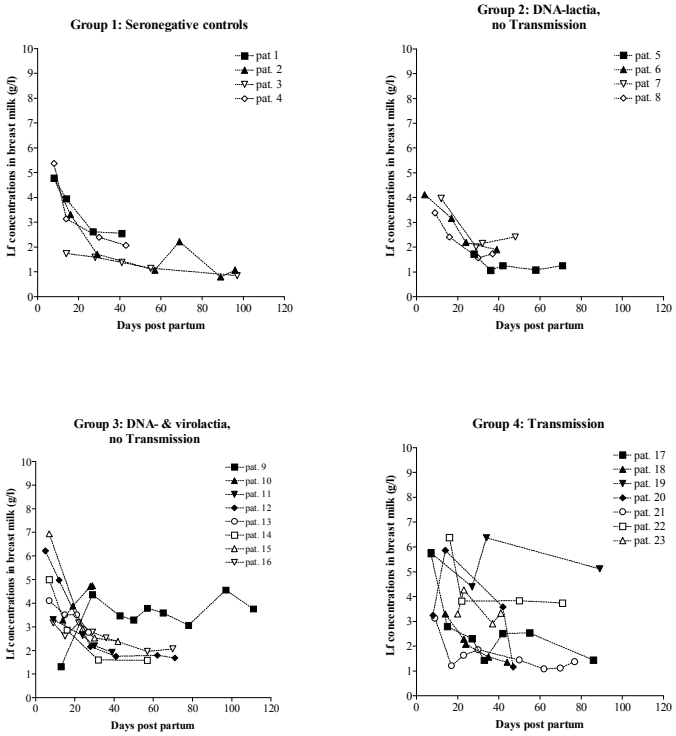

Fig. 1: Lactoferrin concentrations in breast milk decrease during lactation. *Using smoothing spline fitting, no significant differences in decline or initial altitude of LF levels in mature milk were observed.*

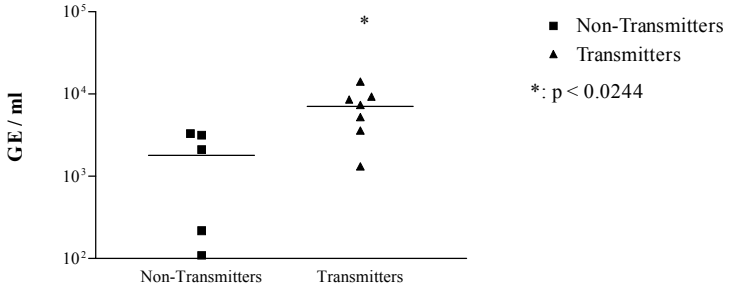

Fig. 2: Quantification of viral DNA in milk whey of maternal HCMV transmitters and non-transmitters. *HCMV DNA load in milk whey of maternal transmitters is significantly increased, as compared to non-transmitters.*

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